ELSEVIER

Molecular and Cellular Probes 18 (2004) 45–50

MOLECULAR AND CELLULAR PROBES

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Inhibition of real-time RT–PCR quantification due to tissue-specific contaminants

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Received 5 March 2003; accepted for publication 5 September 2003

Abstract

Real-time reverse transcription–polymerase chain reaction (RT–PCR) is currently considered the most sensitive method to study low abundance gene expression. Since comparison of gene expression levels in various tissues is often the purpose of an experiment, we studied a tissue-linked effect on nucleic acid amplification. Based on the raw data generated by a LightCycler instrument, we propose a descriptive mathematical model of PCR amplification. This model allowed us to study amplification kinetics of four common housekeeping genes in total RNA samples derived from various bovine tissues. We observed that unknown tissue-specific factors can influence amplification kinetics but this affect can be ameliorated, in part, by appropriate primer selection.

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Keywords: Quantitative polymerase chain reaction; Real-time reverse transcription–polymerase chain reaction; Gene expression; Housekeeping genes; Ubiquitin; β-actin; GAPDH; 18S rRNA

1. Introduction

Reverse transcription–polymerase chain reaction (RT–PCR) is the method of choice for quantifying low abundant mRNAs in material such as cells and tissues [1–4]. This method is fast and highly reproducible. Further, its high sensitivity is its principal advantage over other techniques.

In real-time PCR the quantification takes place within an exponential phase of the amplification curve [5]. A crossing point (CP) or threshold cycle (Ct) is then extrapolated to determine a starting amount of template molecules. The CP gives the researcher the first raw information about the expression level of a given gene.

All methods of gene quantification report their findings relative to a measurable base (e.g. copies per cell, weight of tissue, volume of blood, etc.). The correct choice of the denominator depends on the question asked and can significantly affect the quality of the results [6]. To obtain an actual number of copies, various 'absolute' standards are often employed [7–9], but even in these cases, the quantification is always relative as some errors in a protocol are inevitably present [6,10]. So called housekeeping or maintenance genes [11] such as actins, tubulins, albumins, ubiquitin, glyceraldehyd-3-phosphate dehydrogenase (GAPDH), 18S or 28S ribosomal subunits (rRNA) are often used as relative standards [12]. These genes are believed to undergo little, if any, variation in expression under most experimental treatments. Yet, there have been many reports on the regulation of these genes [12–14].

Another important criterion for reliable measurement and comparison of more than one gene is that all of the genes amplify equally. Experiments using normalization with housekeeping genes often overlook this parameter despite the fact that corrections have already be suggested in the literature [15–19].

Many factors present in samples as well as exogenous contaminants have been shown to inhibit PCR (review in Refs. [20,21]). For example, the presence of hemoglobin, fat, glycogen, cell constituents, Ca^{2+}, DNA or RNA concentration, and DNA binding proteins are important factors [20,21]. Additionally, exogenous contaminants such as glove powder and phenolic compounds from

Abbreviations: RT–PCR, reverse transcription–polymerase chain reaction; CP, crossing point; GAPDH, glyceraldehyd-3-phosphate dehydrogenase; FDM, first derivative maximum; SDM, second derivative maximum.

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doi:10.1016/j.mcp.2003.09.001

the extraction process or the plastic ware can have an inhibiting effect [20,21].

Since some experiments compare gene expression in different organs [9,22], tissue-specific inhibition of DNA amplification may be important. To study the amplification inhibition associated with three randomly chosen tissue types we proposed a mathematical model describing the DNA amplification kinetics in real-time PCR. Using this model we could compare parameters of the amplification kinetics and analyze them statistically.

2. Materials and methods

2.1. Preparation of cDNA samples

Samples of cerebellum, muscle and liver were gathered from six slaughtered cows, immediately frozen in liquid nitrogen and then stored at −80 °C until the total RNA extraction procedure was performed.

Tissue samples were homogenized and total RNA was extracted with a commercially available product, peqGOLD TriFast (Peqlab, Erlangen, Germany), utilizing a single modified liquid separation procedure [23]. No additional purification was performed. Constant amounts of 1000 ng of RNA were reverse-transcribed to cDNA using 200 units of MMLV Reverse Transcriptase (Promega, Mannheim, Germany) according to the manufacturers instructions.

Integrity of the DNA was determined by electrophoresis on 1% agarose gels. Nucleic acid concentrations were measured on a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) at $OD_{260\ nm}$ with 220–1600 nm UVettes (Eppendorf). Purity of the RNA extracted was determined as the $OD_{260\ nm}/OD_{280\ nm}$ ratio with expected values between 1.8 and 2.0 (BioPhotometer). A possible trend between the samples and their $OD_{260\ nm}/OD_{280\ nm}$ values was examined.

2.2. Real-time PCR fluorescence data acquisition

Primer sequences of four common housekeeping genes; ubiquitin, β-actin, GAPDH and 18S rRNA were designed to span at least one intron (except for 18S rRNA) and synthesized commercially (MWG Biotech, Ebersberg, Germany) as shown in Table 1. PCR conditions were optimized on a gradient cycler (T-Gradient, Biometra, Göttingen, Germany) and subsequently on a LightCycler (Roche Diagnostic, Mannheim, Germany) [24] by analyzing the melting curves of the products [25]. Real-time PCR using SYBR Green I technology [26] on the LightCycler was then carried out to amplify cDNAs from the tissue samples.

Master-mix for each PCR run was prepared as follows: 6.4 μl of water, 1.2 μl $MgCl_2$ (4 mM), 0.2 μl of each primer (4 pmol), 1.0 μl Fast Start DNA Master SYBR Green I mix (Roche Diagnostics). Finally, 9 μl of master-mix and 25 ng of reverse transcribed total RNA in 1 μl water were transferred into capillaries (end volume 10 μl).

The following amplification program was used: After 10 min of denaturation at 95 °C, 40 cycles of real-time PCR with three-segment amplification were performed with: 15 s at 95 °C for denaturation, 10 s at respective annealing temperature (Table 1) and 20 s at 72 °C for elongation. A melting step was then performed with slow heating starting at 60 °C with a rate of 0.1 °C/s up to 99 °C with continuous measurement of fluorescence. The same gene was always quantified in each run to prevent any inter-run variation.

Fluorescence data from real-time PCR experiments were taken directly from LightCycler software version 3 (Roche Diagnostics), exported to SigmaPlot 2000 (SPSS, Munich, Germany) and fitted with a '*Four-parametric sigmoid model*' as described earlier by our group [27]. Parameters a, b, x_0 and y_0 of each fit were documented together with the coefficient of determination r^2.

All statistics were done in SigmaPlot 2000 (SPSS) and SigmaStat 2.0 (SPSS, Jandel Corporation).

2.3. Crossing point (CP) acquisition

On each individual real-time PCR run, five different CPs were acquired based on different determination procedures. First, the CP was placed into the first derivative maximum ($FDM_{SM} = x_0$) and into the second derivative maximum of the four-parametric sigmoid model (SDM_{SM}) of each run as shown earlier [27].

Table 1
Details of primers used to amplify four housekeeping genes

Gene	Primers	Sequence length (bp)	Annealing temperature (°C)
Ubiquitin	for: AGA TTC AGG ATA AGG AAG GCA T rev: GCT CCA CCT CCA GGG TGA T	198	60
GAPDH	for: GTC TTC ACT ACC ATG GAG AAG G rev: TCA TGG ATG ACC TTG GCC AG	197	58
18S rRNA	for: GAG AAA CGG CTA CCA CAT CCA A rev: GAC ACT CAG CTA AGA GCA TCG A	338	60
β-actin	for: AAC TCC ATC ATG AAG TGT GAC G rev: GAT CCA CAT CTG CTG GAA GG	234	60

Table 2
Two-way ANOVA

Factor	a	b	FDM_{SM}	SDM_{SM}	FP_{LC}	SDM_{LC}	CP_{Tm}
Tissue	0.01	<0.001	0.004	0.02	0.005	0.008	0.004
Gene	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Tissue–gene interaction	0.004	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

P-values of significance. Each of three rows indicates either one of factors or their interaction. In columns, *P*-values of effect of factors (or interaction) on respective parameter are shown.

Further, CP was computed using the '*Fit point method*' (FP_{LC}) [5] and '*Second derivative maximum method*' (SDM_{LC}) [5,28], both part of the LightCycler software 3.3 (Roche Diagnostics). In the FP_{LC} method, uninformative background fluorescence observations were discarded by setting a constant noise band. An intersecting line was then arbitrarily placed at the base of the exponential portion of the amplification curves. This generated CPs acquired at a constant fluorescence level (value 2 in our case).

In the SDM_{LC} method the second derivative maximum is calculated by LightCycler software based on an unknown and unpublished mathematical approximation of partial amplification kinetics around the supposed SDM_{LC} [5,28].

The FP_{LC} and SDM_{LC} were directly obtained from the calculated values by the LightCycler software 3.3 (Roche Diagnostics).

Eventually, the '*Taqman threshold level*' (Ct) or CP [29] computing method was simulated by fitting the intersecting line upon the 10 times value of ground fluorescence standard deviation (CP_{Tm}). In the '*Taqman threshold level*' procedure, the y_0 values of the four-parametric sigmoid model were considered ground fluorescence.

While parameters a and b describe amplification kinetics, FDM_{SM}, SDM_{SM}, FP_{LC}, SDM_{LC}, and CP_{Tm} are considered quantification parameters since they are clearly defined constants within the model.

Table 3a
Statistically processed parameters a, b, FDM_{SM}, SDM_{SM}, FP_{LC}, SDM_{LC}, CP_{Tm}, and r^2 of ubiquitin amplification

Tissue		a	b	FDM_{SM}	SDM_{SM}	FP_{LC}	SDM_{LC}	CP_{Tm}	r^2
Cerebellum	Mean	43.118	1.950	25.649	23.082	20.180	21.817	22.680	1.000
	CV (%)	9.56	1.17	1.31	1.50	2.13	1.43	1.77	0.004
Liver	Mean	39.355	2.004	26.184	23.545	20.688	22.288	22.597	1.000
	CV (%)	7.79	1.62	1.43	1.64	1.73	1.79	1.47	0.010
Muscle	Mean	41.958	2.064	26.443	23.725	20.637	22.487	25.370	0.999
	CV (%)	5.40	2.25	0.81	0.94	1.52	1.15	0.67	0.018
$Mean_{total}$		41.477	2.006	26.092	23.450	20.502	22.197	23.549	1.000
$CV_{in-tissue}$ (%)		7.58	1.68	1.18	1.36	1.79	1.46	1.30	0.011
$CV_{out-tissue}$ (%)		4.65	2.85	1.55	1.41	1.36	1.55	6.70	0.014

P-values of significance. Each of three rows indicates either one of factors or their interaction. In columns, *P*-values of effect of factors (or interaction) on respective parameter are shown.

Table 3b
Statistically processed parameters a, b, FDM_{SM}, SDM_{SM}, FP_{LC}, SDM_{LC}, CP_{Tm}, and r^2 of GAPDH amplification

Tissue		a	b	FDM_{SM}	SDM_{SM}	FP_{LC}	SDM_{LC}	CP_{Tm}	r^2
Cerebellum	Mean	47.223	2.075	23.663	20.930	18.185	19.583	20.483	0.998
	CV (%)	11.48	1.43	1.14	1.36	1.90	1.43	2.01	0.009
Liver	Mean	46.675	2.094	24.936	22.179	19.322	20.868	21.580	0.998
	CV (%)	6.39	2.75	1.61	1.97	2.09	2.21	2.20	0.020
Muscle	Mean	52.415	2.228	21.588	18.653	15.800	17.440	16.377	0.997
	CV (%)	3.79	2.94	3.30	4.08	4.70	4.06	4.48	0.032
$Mean_{total}$		48.771	2.132	23.396	20.587	17.769	19.297	19.480	0.998
$CV_{in-tissue}$ (%)		7.22	2.37	2.02	2.47	2.90	2.57	2.89	0.020
$CV_{out-tissue}$ (%)		6.50	3.92	7.22	8.68	10.12	8.98	14.08	0.068

P-values of significance. Each of three rows indicates either one of factors or their interaction. In columns, *P*-values of effect of factors (or interaction) on respective parameter are shown.

Table 3c
Statistically processed parameters a, b, FDM_{SM}, SDM_{SM}, FP_{LC}, SDM_{LC}, CP_{Tm}, and r^2 of 18S rRNA amplification

Tissue		a	b	FDM_{SM}	SDM_{SM}	FP_{LC}	SDM_{LC}	CP_{Tm}	r^2
Cerebellum	Mean	49.782	2.701	15.274	11.717	9.518	10.556	10.923	0.996
	CV (%)	3.76	5.33	3.64	6.26	6.32	5.83	6.17	0.047
Liver	Mean	53.544	2.897	14.669	10.854	8.638	9.809	9.185	0.996
	CV (%)	3.35	2.55	9.01	12.21	12.28	12.38	11.15	0.040
Muscle	Mean	55.943	2.752	15.369	11.744	9.250	10.573	10.267	0.997
	CV (%)	2.67	2.31	5.61	7.76	8.32	7.94	7.75	0.041
$Mean_{total}$		53.090	2.784	15.104	11.439	9.135	10.313	10.125	0.996
$CV_{in\text{-}tissue}$ (%)		3.26	3.40	6.09	8.74	8.97	8.72	8.36	0.042
$CV_{out\text{-}tissue}$ (%)		5.85	3.66	2.51	4.43	4.94	4.23	8.67	0.019

P-values of significance. Each of three rows indicates either one of factors or their interaction. In columns, P-values of effect of factors (or interaction) on respective parameter are shown.

2.4. Statistical evaluation of model parameters

Two-way ANOVA with tissue as the first factor of three levels (cerebellum, muscle and liver) and gene as the second factor of four levels (ubiquitin, β-actin, GAPDH, 18S rRNA) was applied to the parameters a, b, FDM_{SM}, SDM_{SM}, FP_{LC}, SDM_{LC} and CP_{Tm} (Table 2). Normal distribution was given within the data sets.

For all above-mentioned parameters and r^2 following statistical indicators were calculated (Tables 3a–3d)

- Interaction mean (i.e. from the six values within one level of factor gene and one level of factor tissue) and interaction coefficient of variance-CV.
- Total mean ($mean_{total}$) out of 18 values (always six samples in three tissues) for each factor gene.
- Mean value out of three CVs ($CV_{in\text{-}tissue}$) reporting internal variance within all three tissue levels.
- Coefficient of variance out of three interaction means ($CV_{out\text{-}tissue}$) showing a variability caused by factor tissue.

3. Results and discussion

All primers used could satisfactorily amplify the flanked sequence. The melting curve analysis and gel analysis detected very little, if any, nonspecific product. We approximated the PCR amplification kinetics with the four-parametric sigmoid model. This model describes well (in all data sets $r^2 > 0.99, n = 40$) the entire fluorescence curve and therefore its beginning and end do not need to be arbitrarily delimited [19]. Nevertheless, correlation between values of b and r^2 showed that there were differences in the goodness of the fit (Pearson correlation coefficient $r = 0.915, n = 72$). The best fit was in runs with high amplification efficiencies. With decreasing amplification efficiency the determination power of the model also decreased.

There is an integral purification step at the end of the extraction procedure [23], consisting of repeated washing the final total RNA pellet with ethanol. In this study no additional RNA purification was performed since additional purification decreases yield and is often omitted. This procedure simulated a routine PCR sample preparation as it is carried out in most labs. The contamination within the RNA samples detected as $OD_{260\ nm}/OD_{280\ nm}$ ratios was not significantly related to the type of tissue (data not shown).

Statistical analysis of the parameters a and b (Table 2) under an influence of the two experimental factors showed that the tissue was the largest source of variance and the primer sequences had the least affect [21,22].

A similar trend of variability within the log-linear trajectory slope (b) and plateau height (a) showed that the tissue from

Table 3d
Statistically processed parameters a, b, FDM_{SM}, SDM_{SM}, FP_{LC}, SDM_{LC}, CP_{Tm}, and r^2 of β-actin amplification

Tissue		a	b	FDM_{SM}	SDM_{SM}	FP_{LC}	SDM_{LC}	CP_{Tm}	r^2
Cerebellum	Mean	85.015	1.418	22.499	20.632	16.640	19.362	19.643	1.000
	CV (%)	5.11	2.15	2.22	2.54	3.21	2.69	2.34	0.004
Liver	Mean	86.694	1.467	23.555	21.624	17.400	20.348	18.633	1.000
	CV (%)	2.14	1.31	0.85	0.95	1.52	1.11	1.11	0.002
Muscle	Mean	84.886	1.470	24.264	22.328	18.230	21.047	20.813	1.000
	CV (%)	2.75	3.53	0.90	1.03	1.14	1.16	0.88	0.005
$Mean_{total}$		85.532	1.452	23.440	21.528	17.423	20.252	19.697	1.000
$CV_{in\text{-}tissue}$ (%)		1.00	2.33	1.32	1.51	1.96	1.65	1.45	0.004
$CV_{out\text{-}tissue}$ (%)		1.18	2.01	3.79	3.96	4.56	4.18	5.54	0.001

P-values of significance. Each of three rows indicates either one of factors or their interaction. In columns, P-values of effect of factors (or interaction) on respective parameter are shown.

which total RNA was extracted has a significant effect on the PCR kinetics and thus on the CP acquisition (Table 2). This can be caused by different amounts of cellular debris present in samples after RNA extraction [30,31]. Also endogenous contaminants such as blood or fat play an important role. Contamination of the sample may affect both the PCR as well as the preceding RT reaction [20,21].

Since interaction between both factors; tissue and gene is significant, the tissue-specific disturbance is not the same for all four amplified sequences but rather is sequence-specific. In our study, the highest resistance to tissue-specific disturbance showed the sequence of β-actin followed by ubiquitin, 18S rRNA and GAPDH (see $CV_{out-tissue}$ values in Tables 3a–3d). A plausible explanation of this interaction may be the presence of specific DNA blocking by polysaccharides or proteins present as endogenous contaminants in the sample [32]. It is possible that DNA amplification may be affected by regions of the template DNA that are specifically blocked by these endogenous macromolecules. Our data show that not only the choice of housekeeping genes [12–14] but also tissue-specific factors and the sequence-specific factors can affect the expression assays.

Tissue-specific suppression can be compensated, in part, by well performing primers such as those for β-actin and ubiquitin used here. From this data it seems that sequences that amplified with higher efficiency (i.e. small *b*) better resist inhibition and show lower variance in all parameters of the PCR kinetics (compare $mean_{total}$ of *b* and $CV_{out-tissue}$ values in Tables 3a and 3d with Tables 3b and 3c). Thus, primer selection and documenting the reaction efficiency are important PCR optimization steps. Although house-keeping genes are expressed differently in various tissues our data show that some vary less than others. For example, ubiquitin showed marginally higher variance between tissues than within one tissue (compare $CV_{out-group}$ with $CV_{in-group}$ in Table 3a). This suggests that the expression of ubiquitin in the different tissues was similar. The low variance for ubiquitin expression between tissues suggests that it is the best standard but is closely followed by β-actin and GAPDH. 18S rRNA, with its high variance, seems to be less suitable as an internal standard. This order was preserved in all CP computing methods.

Each method of computing CPs seems to be accurate for estimating expression levels but they varied slightly when CP acquisitions took place at different heights of the amplification curve (Tables 3a–3d). The method of first and second derivative maximum computed from the four-parametric sigmoid model is reliable and simple and generates reliable CPs comparable with other methods (see CV values in Tables 3a–3d).

Acknowledgements

The experimental animals were slaughtered at the EU-official slaughterhouse of the Bayerische Landesanstalt für Tierzucht at Grub, 85580 Poing, Germany.

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